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Diversity of the Bacterial Community in the Rice Rhizosphere Managed Under Conventional and No-tillage Practices[§]

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Bacterial diversity in the rice rhizosphere at different rice growth stages, managed under conventional and no-tillage practices, was explored using a culture-based approach. Actinobacteria are among the bacterial phyla abundant in the rice rhizosphere. Their diversity was further examined by constructing metagenomic libraries based on the 16S rRNA gene, using actinobacterial- and streptomycete-specific polymerase chain reaction (PCR) primers. The study included 132 culturable strains and 125 clones from the 16S rRNA gene libraries. In conventional tillage, there were 38% *Proteobacteria*, 22% *Actinobacteria*, 33% *Firmicutes*, 5% *Bacteroidetes*, and 2% *Acidobacteria*, whereas with no-tillage management there were 63% *Proteobacteria*, 24% *Actinobacteria*, 6% *Firmicutes*, and 8% *Bacteroidetes* as estimated using the culture-dependent method during the four stages of rice cultivation. Principal coordinates analysis was used to cluster the bacterial communities along axes of maximal variance. The different growth stages of rice appeared to influence the rhizosphere bacterial profile for both cultivation practices. Novel clones with low similarities (89–97%) to *Actinobacteria* and *Streptomyces* were retrieved from both rice fields by screening the 16S rRNA gene libraries using actinobacterial- and streptomycete-specific primers. By comparing the actinobacterial community retrieved by culture-dependent and molecular methods, it was clear that a more comprehensive assessment of microbial diversity in the rice rhizosphere can be obtained using a combination of both techniques than by using either method alone. We also succeeded in culturing a number of bacteria that were previously described as unculturable. These were in a phylogenetically deep lineage when compared with related cultivable genera.

Keywords: rice, no-tillage, diversity, phylogeny, actinobacteria

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Introduction

Rice (*Oryza sativa* L.) is a major staple food for nearly two-thirds of the world's population, particularly in Asia. In Korea, paddy fields make up about 61% of the total agricultural area, and environmentally sustainable farming technology has been attempted during the last decade to replace existing farm machinery. Among the sustainable farming technologies, a no-tillage practice has been introduced for rice production in some areas (Choe *et al*., 1998).

No-tillage systems result in significant increases in soil organic carbon, total nitrogen levels, and microbial biomass at the surface layer by improving soil quality (Bhattacharyya *et al*., 2009). In contrast, conventional tillage decreases soil organic matter by affecting the physiological and chemical properties of the soil environment, as well as the microclimate that regulates biological processes. Soil microbial communities mediate many processes essential to the productivity and sustainability of soil, and their structure becomes simplified by intensive tillage resulting in lower stability of biochemical functions in soil (Feng *et al*., 2003). A few studies have been conducted to analyze the bacterial communities of rice paddy soils by using environmental sequences of the 16S rRNA gene and culturable bacteria (Chin *et al*., 1999). Microbial diversity of methanogenic or methanotrophic communities in paddy soils have recently been analyzed by molecular and phospholipid biomarker methods (Horz *et al*., 2001). However, other bacterial phyla such as *Actinobacteria* have not received much attention in rice paddy soils, although they have been studied intensively in the rhizospheres of other cereal crops (Coombs and Franco, 2003). Molecular techniques have been used to detect *Actinobacteria* in environmental samples when culture-based methods were unsuccessful (Rheims and Stackebrandt, 1999). *Actinobacteria* are important because they play a significant role in agriculture, ecology, industry, and medicine (McNeill and Brown, 1994). Therefore, there should be a proper investigation of their diversity, species richness and distribution.

Rice has different growth stages including vegetative, reproductive, and ripening during which different physiological and biochemical activities occur (Hua *et al*., 2006). These activities definitely affect the rhizosphere microbial communities. Several studies have been conducted to observe changes in the microbial communities during the different

rice growth stages, and these changes may be due to the unique oxic and anoxic environments of rice paddy soils (Reichardt *et al.*, 1997). This might make it more difficult to analyze diverse culturable and unculturable bacteria present at a relatively high abundance. Unculturable bacteria are estimated to be >99% of all bacterial communities based on metagenomic analysis of environmental samples (Amann *et al.*, 1995), and difficulties in isolating and characterizing these unculturable bacteria are a major barrier. To overcome this barrier, different isolation methodologies have been attempted (using low nutrient media, sonication, addition of soil extracts, long incubation times and using culture environments replicating the natural environment from which samples were taken), with some success (Zengler *et al.*, 2002; Davis *et al.*, 2005).

In the present study, the effects of conventional and no-tillage practices on bacterial communities of rhizosphere soil were determined during rice cultivation by analyzing the bacterial community structure using a culture-dependent method. The actinobacterial community was also analyzed by constructing metagenomic libraries based on actinobacterial- and streptomycete-specific primers.

Materials and Methods

Sample collection and chemical analysis

This study was performed on a 5-year-old experimental rice field established at the Gyeongsang National University farm (Daegok valley, 35° 14′ 21″ N, 128° 13′ 23″ E), Jinju, Korea. The climate was subtropical with an average annual temperature of 14°C (October to March, 6.8°C; April to September, 21.3°C) and annual rainfall of 1,701 mm in 2007. The experiment was established in such a way that the conventional tillage (T) field was under a normal cultivation system, whereas the no-tillage (Z) field was kept without plow disturbance for at least 5 years. Both fields were under a monoculture system and separated by a 3 m wide footpath. The rice (*Oryza sativa*, Japonica type, variety Ilmi-byo) was grown from seedlings with a drill in the T field, whereas the seeds were spread manually in the flooded condition in the Z field. General cultivation methods were used according to the standard rice cultivation protocol provided by the National Institute of Crop Science, RDA, Korea (http//www.nics.go.kr/Crop Webzine). The soil chemical properties during the experiment are shown in Table 1. Soil samples were collected from the rice fields at four different growth stages: pre-sowing (0), vegetative (1, 1 week after transplanting), reproductive (2, tillering and flowering) and ripening (3, seeds fully matured). T0, T1, T2, and T3 represent the samples taken from the conventional tillage field, whereas Z0, Z1, Z2, and Z3 represent the samples taken from the no-tillage field at the four stages of rice growth. The first and last stage samples were taken under dry field conditions, whereas the second and third stage samples were collected under flooded conditions, both in 2007. Each sample was comprised of five sub-samples collected at a depth of 0–20 cm and a width of 6–10 cm (or as far as the roots were spread) from five randomly selected points in each field after removing the upper 2–3 cm of soil, some straw, and debris. The five sub-samples were combined and processed immediately for culturing upon reaching the laboratory. A part of the samples was stored at 4°C for chemical analysis.

The samples for DNA isolation were stored at -70°C. The pH of the samples was measured using a digital pH meter in a 1/10 (w/v) aqueous solution using distilled water. Total carbon was determined by the partial-oxidation method and total nitrogen was measured by the micro Kjeldahl method (Bremner and Mulvaney, 1982). Mean values of the soil characteristics were calculated for data from the T and Z rhizosphere soils collected from eight different samples at each growth stage. The values between two samples were compared by one-way analysis of variance using SPSS 14.0 software (SPSS Inc., USA).

Isolation and cultivation of bacteria

One gram of soil was added to 10 ml of 50 mM phosphate buffer (pH 7.0) and 50% of the soil mixture was treated by sonication with an electronic homogenizer (Bandelin Sonoplus, Berlin, Germany) at 260 W/cm^2 for 15 sec to isolate bacteria from the rhizosphere soil samples. Serial dilutions were made after mixing both sonicated and non-sonicated portions. The diluted aliquots were spread on modified half-strength R2A agar plates (Aslam *et al.*, 2008). A 100 µl aliquot was applied to half-strength R2A agar medium in large polystyrene petri-dishes (15 cm diameter) for 10^{-3} and 10^{-5}dilutions, and the plates were incubated at 28°C for more than 1 month. The isolation medium was supplemented with 40% (v/v) soil extract and 50 µg/ml amphotericin B to inhibit fungal growth. The colonies were selected based on morphology, and the isolates were sub-cultured on modi-

Table 1. Characteristics of soil samples collected from two rice fields managed under conventional tillage (T) and no-tillage (Z) practices

Parameter (unit)	Conventional tillage (T)					No-tillage (Z)				
Surface area (ha)	4					2				
Soil type	Sandy and clay-loamy					Sandy and clay-loamy				
Samples	Zero-phase	Vegetative-phase	Reproductive-phase	Ripening-phase	*p*-value	Zero-phase	Vegetative-phase	Reproductive-phase	Ripening-phase	*p*-value
Sampling dates	19/04/2007	06/07/2007	07/09/2007	04/10/2007		19/04/2007	06/07/2007	07/09/2007	04/10/2007	
pH	6.5 ± 0.15	6.9 ± 0.15	6.6 ± 0.30	6.0 ± 0.20	0.006	5.7 ± 0.10	6.4 ± 0.10	6.4 ± 0.00	6.2 ± 0.10	0.000
Total carbon (%)	0.68 ± 0.01	0.34 ± 0.02	0.46 ± 0.04	0.57 ± 0.01	0.000	0.86 ± 0.03	0.36 ± 0.02	0.43 ± 0.02	0.49 ± 0.01	0.000
Total nitrogen (%)	0.16 ± 0.02	0.15 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.036	0.16 ± 0.01	0.15 ± 0.01	0.17 ± 0.00	0.17 ± 0.00	0.005

fied half-strength R2A agar and later on half-strength R2A agar plates. The single selected colonies were checked for purity by streaking on half-strength R2A agar medium. The purified strains were identified and stored as 20% glycerol stocks at -70°C. The 16S rRNA gene of the isolates was amplified from purified genomic DNA using a set of universal 27F and 1492R bacterial primers (Table 2) and the purified polymerase chain reaction (PCR) product was sequenced by GenoTech Inc. (Daejeon, Korea) (Lane, 1991). The 16S rRNA gene sequences were compiled using SeqMan software (DNASTAR).

Actinobacterial- and streptomycete-specific clone libraries

Amplification of the 16S rRNA gene using the actinobacterial-specific primers S-C-Act_235-a-S-20 and S-C-Act_878-a-A-19 (Table 2) was performed by Touchdown PCR with some modifications (Babalola, 2009). The PCR reactions were set up in 20 μl volumes consisting of 1.5 mM $MgCl_2$, 0.5 mM of each primer, 200 mM of each dNTP, 1.5 U *Taq* DNA polymerase (Fermentas), and 50 ng of metagenomic DNA. The PCR cycling conditions were an initial denaturation at 95°C for 5 min, followed by 10 cycles of denaturation at 94°C for 1 min, annealing at 70°C for 45 sec, and extension at 72°C for 1 min. This was followed by 20 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 45 sec, and extension at 72°C for 1 min. A final extension was performed for 10 min at 72°C. PCR products were electrophoresed on 1% agarose gels in 1.0× TAE buffer containing ethidium bromide (10 μg/ml) and DNA (band size 640–650 bp) was excised from the gel and purified with a Qiagen PCR DNA and Gel Band Purification kit.

The 16S rRNA gene was also amplified using the StrepB and StrepF streptomycete-specific primers (Table 2) directly from the genomic DNA of all individual samples (Reichardt *et al*., 1997). The PCR cycling conditions were an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 90 sec. A final elongation step was performed at 72°C for 10 min. PCR products (band size 1,050–1,074 bp) were electrophoresed on 1.5% agarose gels in 1.0× TAE buffer. After purification of the actinobacterial- and streptomycete- specific primer PCR products with an Intron DNA and PCR purification kit, they were cloned into the T&A vector following the standard protocol supplied by the manufacturer (RBC T&A Cloning Vector Kit). The recombinant colonies were selected using blue/white color screening. Plasmid DNA was extracted and purified using a Plasmid Purification Kit (NucleoGen Inc.) according to the manufacturer's protocol and sequenced using the vector specific primers T13-F and T13-R (Table 2).

Phylogenetic and statistical analysis

The bacterial strains were phylogenetically analyzed and identified based on the partial sequence of the 16S rRNA gene (490 bp) after calculating pair-wise sequence similarities using the global alignment algorithm and the type strains of the validly published species or genera. A primer set of 27F/1492R was used for 16S rRNA gene sequencing, and the bacterial partial sequences were combined in the BioEdit program (Hall, 1999). After editing, a clear part of each sequence (typically 500–600 bp in length) was selected. Chimeric sequences were identified using the CHECK_CHIMERA program in the Ribosomal Database Project (Maidak *et al*., 1997). Sequences were compared to the available databases using the Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations. Sequences from the BLAST search with the greatest similarity to the clone sequences, and representative sequences of Eubacteria, were obtained from GenBank. Multiple alignments were performed with the CLUSTAL_X program (Thompson *et al*., 1997), and gaps were edited in the BioEdit program (Hall, 1999). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). The phylogenetic trees were constructed using a neighbor-joining method (Saitou and Nei, 1987) in the MEGA4 program (Tamura *et al*., 2007) with bootstrap values based on 1,000 replications. The phylogenetic analyses for the libraries of actinobacterial- and streptomycete-specific clones were performed using the same procedure. Principal coordinates analysis (PCoA) was conducted using the online software UniFrac, which employs genetic distances to evaluate the community similarity based on the gene sequence data (Lozupone *et al*., 2006).

Nucleotide sequence accession numbers

Accession numbers of the sequences determined in this study for the cultured strains and actinobacterial- and streptomycete-specific clones are GQ369008-GQ369139, GQ369150-GQ369222, and GQ369223-GQ369274, respectively.

Table 2. Polymerase chain reaction primers used in this study

Primer[a]	Primer sequence (5′→3′)	Reference
27F	AGAGTTTGATCMTGGCTCAG	Lane (1991)
1492R	TACGGYTACCTTGTTACGACTT	Lane (1991)
S-C-Act_235-a-S-20	CGCGGCCTATCAGCTTGTTG	Stach *et al.* (2003)
S-C-Act_878-a-A-19	CCGTACTCCCCAGGCGGGG	Stach *et al.* (2003)
StrepF	ACGTGTGCAGCCCAAGACA	Rintala *et al.* (2001)
StrepB	ACAAGCCCTGGAAACGGGGT	Rintala *et al.* (2001)
M13-F	CCCAGTCACGACGTTGTAAAACG	
M13-R	AGCGGATAACAATTTCACACAGG	

[a] F, forward primer; R, reverse primer.

Results

Chemical analysis of soil samples

The pH of the T and Z soils increased initially from 6.5 to 6.9 and from 5.7 to 6.4, respectively, with rice cultivation. It decreased from 6.9 to 6.0 in T soils and remained at 6.2–6.4 in Z soils after the vegetative phase (Table 1). Total carbon in T soil decreased from 0.68 to 0.34% during the first two (pre-sowing and vegetative) stages and then increased to 0.46 and 0.57% in later (reproductive and ripening) stages, whereas in the Z soil it decreased from 0.86 to 0.36% during the former two stages and then increased to 0.43 and 0.49% in later stages (Table 1). No significant changes in total nitrogen content were observed during the different growth stages.

Diversity of culturable bacteria, phylogenetics, and the PCoA

Bacterial diversity in two rice fields managed under conventional and no-tillage practices was determined during pre-sowing, vegetative, reproductive, and ripening stages. Among 132 isolated bacterial strains (60 strains from T soil, 72 strains from Z soil), there were 38% *Proteobacteria*, 22% *Actinobacteria*, 33% *Firmicutes*, 5% *Bacteroidetes*, and 2% *Acidobacteria* in the T soil, whereas there were 63% *Proteobacteria*, 24% *Actinobacteria*, 6% *Firmicutes*, and 8% *Bacteroidetes* in the Z soil during the four rice cultivation stages (Fig. 1 and Table 3). For the 132 isolated strains, the abundant families under tillage conditions were *Paenibacillaceae* (15%), *Bacillaceae* (13%), *Sphingomonadaceae* (10%), *Intrasporangiaceae* (7%), *Xanthomonadaceae* (7%), *Methylobacteriaceae* (7%), *Microbacteriaceae* (5%), *Caulobacteraceae* (5%), *Sphingobacteriaceae* (3%), *Micrococcaceae* (3%), *Burkholderiales* incertae sedis (3%), and *Staphylococcaceae* (3%). Under no-tillage conditions, the families were *Bradyrhizobiaceae* (19%), *Xanthomonadaceae* (14%), *Sphingomonadaceae* (13%), *Intrasporangiaceae* (7%), *Microbacteriaceae* (6%), *Bacillaceae* (4%), *Flavobacteriaceae* (4%), *Phyllobacteriaceae* (4%), *Cellulomonadaceae* (3%), *Chitinophagaceae* (3%), *Mycobacteriaceae* (3%), and *Pseudomonadaceae* (3%). Differences in composition of bacteria were observed during the different growth stages. The number of *Proteobacteria* increased from the pre-sowing to the vegetative phase and then remained constant until the ripening phase in both fields. In particular, the number of *Actinobacteria* decreased gradually with cultivation time and the compositions were 32% (Z0), 31% (Z1), 0% (Z2), and 10% (Z3) in the no-tillage soils and 26% (T0), 30% (T1), 21% (T2), and 0% (T3) in the conventional tillage soils. The *Firmicutes* group was also affected by tillage practices and growth stages of rice showing 5% (Z0), 0% (Z1), 0% (Z2), and 20% (Z3) in no-tillage soils and 52% (T0), 0% (T1), 21% (T2), and 33% (T3) in conventional tillage soils. The *Bacteroidetes* group was found at pre-sowing (5%), reproductive (27%) and ripening stages (10%) in the no-tillage soils and at the pre-sowing phase (11%) in conventional tillage soils (Fig. 1). All strains isolated from both T and Z soils were identified based on the partial sequences of the 16S rRNA gene and were analyzed phylogenetically. Of the 132 strains, 102 (77%) had 98.0–100%, 22 (17%) had 96.0–98.0%, four (3%) had 94.0–96.0%, and four (3%) had 88.5–91.5% 16S rRNA gene sequence similarity with the type strains of the related genera (Table 3). The major genera (>5%) in T soils were *Bacillus*, *Brevibacillus*, *Methylobacterium*, *Microbacterium*,

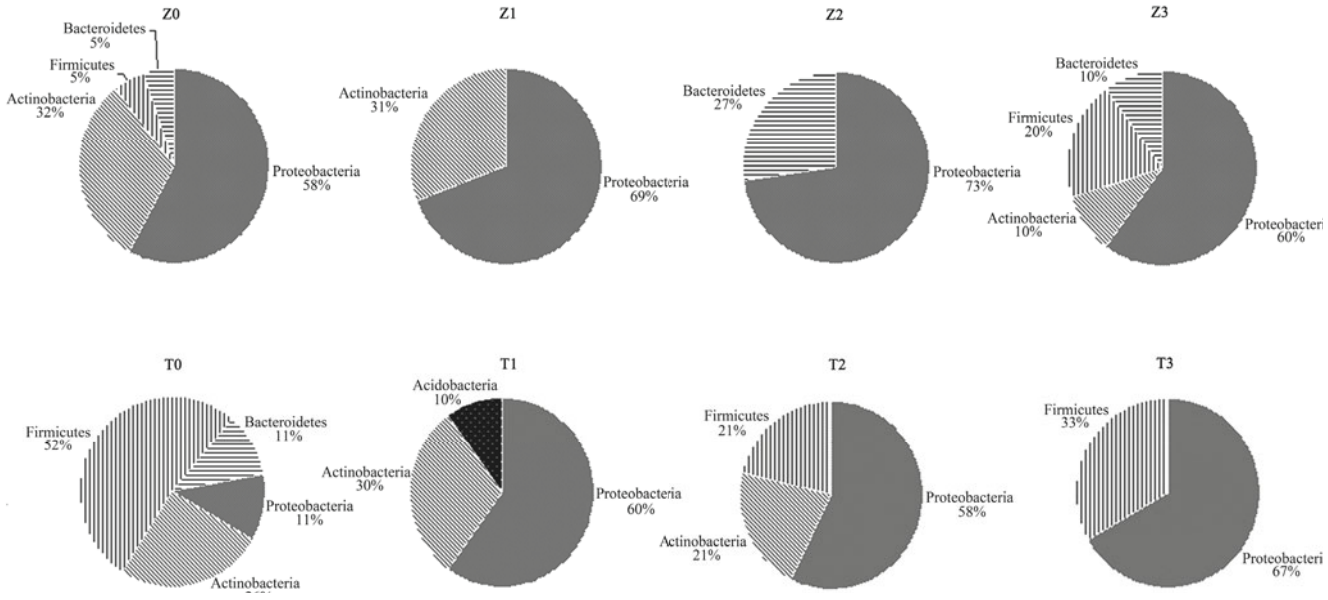

Fig. 1. Comparison of cultured bacterial strains isolated from two rice fields managed under conventional tillage and no-tillage. T0–T3 and Z0–Z3 represent samples taken from the two rice fields managed under the conventional and no-tillage practices at four stages, namely pre-sowing (0), vegetative (1), reproductive (2), and ripening (3), respectively. The bacterial groups are represented in percentages. The total number of strains isolated from conventional and no-tillage fields during the four stages are T0 = 27, T1 = 10, T2 = 14, T3 = 9, and Z0 = 38, Z1 = 13, Z2 = 11, and Z3 = 10, respectively.

Table 3. Comparison of the strains isolated from two rice fields managed under conventional tillage (T) and no-tillage (Z) practices using the culture-dependent method

Phylum	Closely related genus	No. of strains from conventional tillage (T)	No. of strains from no-tillage (Z)	Similarity (%)[a]
Acidobacteria	*Geothrix*	1	0	97.31
Actinobacteria	*Ilumatobacter*	1	0	91.86
	Mycobacterium	0	2	98.95–98.97
	Rhodococcus	0	1	98.99
	Cellulomonas	0	1	97.34–99.21
	Humibacillus	0	1	99.85
	Phycicoccus	0	1	99.64
	Terrabacter	4	3	97.52–100
	Leifsonia	0	1	99.36
	Microbacterium	3	2	99.86–98
	Plantibacter	0	1	99.85
	Arthrobacter	2	1	98.65–100
	Micromonospora	1	0	98.62
	Micromonospora	0	1	98.44
	Nocardioides	1	0	98.84
	Streptomyces	1	1	98.28–100
Bacteroidetes	*Spirosoma*	1	0	100.00
	Chryseobacterium	0	1	98.73
	Flavobacterium	0	2	98.77–97.91
	Flavobacterium	0	1	97.91
	Chitinophaga	0	2	97.95–98.06
	Pedobacter	1	0	97.71
	Pedobacter	1	1	97.25–98.35
Firmicutes	*Bacillus*	7	3	99.53–100
	Brevibacillus	3	1	98.08–99.92
	Paenibacillus	7	0	97.50–100
	Psychrobacillus	1	0	99.26
	Staphylococcus	2	0	99.72–99.9
Proteobacteria; Alphaproteobacteria	*Afipia*	0	2	91.35–91.51
	Altererythrobacter	0	1	97.07
	Caulobacter	1	0	99.05
	Phenylobacterium	2	0	99.52–97.78
	Bradyrhizobium	1	12	96.87–99.55
	Rhodoplanes	0	1	93.74
	Methylobacterium	4	0	99.24–100
	Mesorhizobium	0	3	99.48-99.82
	Rhizobium	0	1	98.60
	Roseomonas	1	0	96.21
	Dongia	1	0	95.52
	Sphingomonas	6	9	98.36–100
Proteobacteria; Betaproteobacteria	*Burkholderia*	0	1	99.83
	Variovorax	0	1	97.66
	Pseudoduganella	0	1	98.54
	Cupriavidus	1	0	99.64
	Ideonella	1	1	97.79
	Polyangium	1	0	96.24
	Rhizobacter	0	2	97.56–99.67
Proteobacteria; Gammaproteobacteria	*Arenimonas*	0	1	100.00
	Dyella	0	2	99.19–99.73
	Lysobacter	0	1	99.25
	Lysobacter	0	3	99.03–100.00
	Pseudoxanthomonas	1	1	99.71
	Rhodanobacter	3	0	98.64–99.27
	Rudaea	0	1	99.56
	Stenotrophomonas	0	1	99.61

[a] Similarity from the identification results using EzTaxon-e database Kim *et al.* (2012).

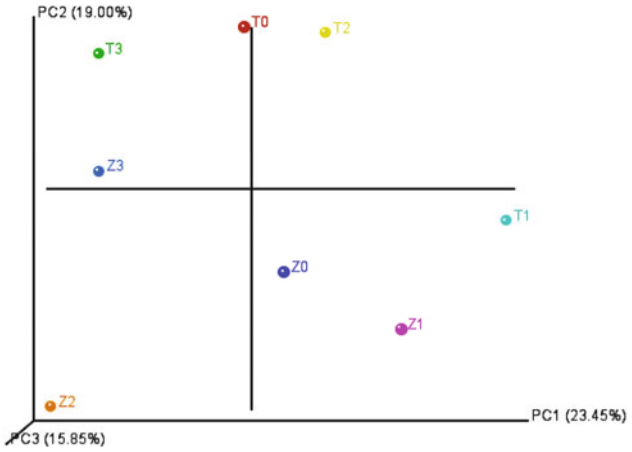

Fig. 2. Principal coordinates analysis of bacterial communities with unweighted UniFrac from rhizosphere soil samples of two rice fields managed under conventional tillage (T) and no-tillage (Z) during four growth stages. The bacterial communities in each phase were roughly clustered by cultivation practice and the maximum variations were 23.45% (PC1) and 15.85% (PC3).

Paenibacillus, *Rhodanobacter*, *Sphingomonas*, *Terrabacter*, whereas those in Z soils were *Bacillus*, *Bradyrhizobium*, *Lysobacter*, *Mesorhizobium*, *Sphingomonas*, and *Terrabacter* (Table 3).

Most of the strains clustered with *Arthrobacter*, *Terrabacter*, *Microbacterium*, *Cellulomonas*, *Mycobacterium*, and *Streptomyces* in the *Actinobacterial* clade, and they were from both field samples and at all growth stages except the ripening stage (Supplementary data Fig. S1). Major clusters in the *Firmicutes* clade were *Panibacillus* and *Bacillus*. The major cluster in the *Alphaproteobacterial* clade was *Sphingomonas*, representing strains from both field samples at almost all stages. Other clusters represented minor groups of strains. However, the six strains with 88.5–96.0% 16S rRNA gene sequence similarity, [Z0-YC6801 (GQ369069), Z0-YC6835 (GQ369103), Z0-YC6837 (GQ369105), Z1-YC6840 (GQ369108), Z2-YC6854 (GQ369122), and Z2-YC6860 (GQ369128)] were isolated from no-tillage soils and had strong lineage association with previously validly published strains that had a high similarity with most of the uncultured bacteria. In particular, five of the 8 strains designated as T2-YC6788, T2-YC6790, Z0-YC6835, Z0-YC6837, and Z2-YC6860 had low 16S rRNA gene sequence similarity ranging from 91.35–95.52% with *Dongia mobilis* LM22^T, *Ilumatobacter fluminis* YM22-133^T, *Afipia birgiae* 34632^T, *Afipia felis* B-91-007352^T, and *Rhodoplanes elegans* AS130^T, respectively (Supplementary data Fig. S1).

PCoA was performed to compare the overall composition of the bacterial communities at the four different growth stages in the rhizosphere of rice managed under conventional tillage and no-tillage practices. PCoA was used to cluster the bacterial communities along axes of maximal

Table 4. Comparison of *Actinobacteria* clones obtained from the metagenomic libraries of two rice fields managed under conventional tillage (T) and no-tillage (Z) practices

Phylum	Closely related genus	No. of clones from conventional tillage (T)	No. of clones from no-tillage (Z)	Similarity (%)[a]
Actinobacteria	*Aciditerrimonas*	3	1	91.84–92.89
	Iamia	0	2	90.47–92.27
	Ilumatobacter	1	1	92.15-93.75
	Corynebacterium	0	2	97.88–98.18
	Mycobacterium	1	0	96.94
	Blastococcus	0	2	97.22–97.58
	Jatrophihabitans	1	0	96.21
	Kineosporia	1	0	97.87
	Janibacter	1	0	97.13
	Phycicoccus	1	0	97.71
	Terrabacter	2	1	97.88–98.64
	Salinibacterium	1	0	97.58
	Arthrobacter	5	12	95.61–98.79
	Actinocatenispora	1	0	89.56
	Actinoplanes	0	1	98.49
	Kribbella	1	1	98.47–98.79
	Marmoricola	1	0	96.68
	Nocardioides	4	3	96.78–98.34
	Microlunatus	0	1	95.14
	Propionibacterium	1	1	97.12-97.88
	Actinosynnema	1	1	93.94
	Streptomyces	2	5	89.09–98.79
Firmicutes	*Gracilibacter*	0	1	84.46
Gemmatimonadetes	*Gemmatimonas*	6	4	88.79–91.2

[a] Similarity from the identification results using EzTaxon-e database Kim *et al.* (2012).

variance. PC1 and PC2 explained 23.45% and 19.0% of the variation, respectively, in the data. The third PC contributed 15.85% of the observed variation. The different rice growth stages appeared to influence the rhizosphere bacterial profile between the two cultivation practices (Fig. 2). Bacterial communities of T0, T2, and T3 of the tillage and Z0, Z1, and Z2 of the no-tillage soils were separated along the second PC, whereas T1 and Z3 differed from each other particularly along the first PC. Similar to the PCoA results, the Jackknife environment cluster results suggested that the T0, T2, T3, and Z3 were more similar to each other than they were to sequences from the Z0, Z1, and Z2. Moreover, T1 clustered with both T0 and Z0. Performing the P test for significance and UniFrac significance at all time points of the growth phase together with the number of permutations set to 1,000 resulted in a significant *P*-value for the *P*-test (P=0.001) and a significant *P*-value for UniFrac significance (P=0.04). This result indicates that the sequences were significantly clustered by growth stages in each cultivation practice. We performed a lineage-specific analysis with a branch length threshold of 0.317 to separate the tree into lineages that were at or slightly above the family level to identify lineages that contributed to the differences between growth stages. Only one node showed a significant G test *P*-value of 0.008 indicating that the node had an excess or deficit of sequences in a particular growth phase relative to chance expectations. We determined that the node represented species of *Bacilli*. Comparing the observed and expected counts demonstrated that there were more *Bacilli* sequences in the T0, T2, and Z0 than expected, and fewer in the other growth stages of rice (Fig. 2).

Actinobacterial- and streptomycete-specific clones and their phylogenetic analysis

A total of 73 clones (7–10 clones per sample) were screened with actinobacterial-specific primers by randomly picking white colonies among the blue ones, then identifying them on the basis of 16S rRNA gene sequence and analyzing them phylogenetically. The distribution of these groups are listed in Tables 4 and 5. Major genera (>5%) in T soils that belonged to the phylum *Actinobacteria* were *Arthrobacter*, *Aciditerrimonas*, *Nocardioides*, *Streptomyces*, and *Terrabacter*, whereas those in Z soils were *Arthrobacter*, *Blastococcus*, *Corynebacterium*, *Iamia*, *Nocardioides*, and *Streptomyces*. The major genus *Gemmatimonas* belonging to the phylum Gemmatimonadetes appeared at 17.6% in T soil and 10.3% in Z soil samples.

A neighbor-joining phylogenetic tree of the 73 clones (614 bp) with the most closely related type strains was constructed (Supplementary data Fig. S2). *Gemmatimonas aurantiaca*

Table 5. List of *Streptomyces* clones obtained from the metagenomic libraries of two rice fields managed under conventional tillage (T) and no-tillage (Z) practices

Most related species	No. of clones from conventional tillage (T)	No. of clones from no-tillage (Z)	Similarity (%)[a]
Kitasatospora paracochleata IFO 14769^T	1	0	98.29
Streptomyces alanosinicus NBRC 13493^T	0	1	97.79
Streptomyces albidoflavus DSM 40455^T	0	1	98.35
Streptomyces avermitilis MA-4680^T	1	0	98.12
Streptomyces bungoensis NBRC 15711^T	1	0	99.04
Streptomyces cacaoi subsp. *asoensis* NRRL B-16592^T	0	1	98.31
Streptomyces canus NRRL B-1989^T	1	0	97.84
Streptomyces chrysomallus subsp. *fumigatus* NBRC 15394^T	1	0	97.62
Streptomyces cirratus NRRL B-3250^T	1	2	99.11–99.44
Streptomyces coacervatus AS-0823^T	0	1	98.24
Streptomyces diastatochromogenes ATCC 12309^T	2	0	98.53–98.89
Streptomyces exfoliatus NBRC 13191^T	1	0	97.62
Streptomyces gramineus JR-43^T	9	3	95.85–98.13
Streptomyces griseoruber NBRC 12873^T	7	3	97.62–98.98
Streptomyces lincolnensis NRRL 2936^T	1	0	97.53
Streptomyces mirabilis NBRC 13450^T	1	0	97.98
Streptomyces olivochromogenes NBRC 3178^T	1	0	98.77
Streptomyces phaeopurpureus NRRL B-2260^T	0	1	98.25
Streptomyces psammoticus NBRC 13971^T	2	1	98.23–98.95
Streptomyces recifensis NBRC 12813^T	1	0	98.07
Streptomyces rishiriensis NBRC 13407^T	3	0	98.04–98.84
Streptomyces shenzhenensis 172115^T	0	1	98.12
Streptomyces xanthocidicus NBRC 13469^T	1	0	98.41
Streptomyces yanii NBRC 14669^T	0	1	98.98
Streptomyces yerevanensis NBRC 12517^T	0	1	99.89
Total	35	17	

[a] Similarity from the identification results using EzTaxon-e database Kim *et al.* (2012).

T-27^T was used as the out-group and one Z0A-06 clone (GQ369189) was discarded due to short sequence length. *Arthrobacter* was the most common group, accounting for 23% of the total actinobacterial clones (17 clones), followed by *Streptomyces* (seven clones with 89.1–98.8% sequence similarity), *Nocardioides* (seven clones with 96.8–98.3% sequence similarity), *Aciditerrimonas* (four clones with 91.8–92.9% sequence similarity), and *Terrabacter* (three clones with 97.9–98.6% sequence similarity). Other minor groups had low sequence similarity.

Non-specific binding of actinobacterial-specific primers occurred and they amplified a new group of bacteria that was deeply aligned with *Gemmatimodadetes*. Eleven clones including Z0A-09 (GQ369211) were deeply aligned with *Gracilibacter thermotolerans* JW/YJL-S1^T and *Gemmatimonas aurantiaca* T-27^T, which does not belong to *Actinobacteria*. Other clones belonged to different species of *Streptomyces*, such as *S. griseoplanus* NBRC 12779^T, *S. rishiriensis* NBRC 13407^T, *S. griseoaurantiacus* NBRC 15440^T, and *S. globosus* LMG 19896^T of which most had high 16S rRNA gene sequence similarity (98.3–98.6%) except the clone T1A-02 with very low 16S rRNA gene sequence similarity (89.1%) with *S. globosus* LMG 19896^T. However, it was deeply aligned with clone Z1A-8 and *Nocardioides pyridinolyticus* OS4^T. Some specific bacterial clones such as Z1A-05, Z2A-07, and T0A-02 did not seem to be phase-dependent and appeared randomly in both field soils. They were closely related with *Actinosynnema pretiosum* subsp. *auranticum* NBRC 15620^T and *Actinoplanes deccanensis* IFO 13994^T.

Fifty-two clones were selected for sequencing and phylogenetic analysis with the streptomycete-specific primers. A neighbor-joining phylogenetic tree of the clones (507 bp) with the most closely related type strains was constructed (Supplementary data Fig. S3). Most clones (60%) retrieved from both field soils had high sequence similarity (97.8–98.1%) with *Streptomyces gramineus* JR-43^T and formed a large clade with *S. psammoticus* NBRC 13971^T, *S. gramineus* JR-43^T, and *S. shenzhenensis* 172115^T.

The *Streptomyces gramineus* JR-43^T, *S. griseoruber* NBRC 12873^T, and *S. rishiriensis* NBRC 13407^T were three major species at 25.7%, 20.0%, and 8.6% abundance, respectively, in the T soil. In the Z soil the major species were *S. gramineus* JR-43^T, *S. griseoruber* NBRC 12873^T, and *S. cirratus* NRRL B-3250^T at 17.6%, 17.6%, and 11.8%, respectively (Table 5). No non-specific binding of streptomycete-specific primers was observed.

Discussion

Diverse bacterial communities in the rhizosphere soil of rice fields managed under conventional and no-tillage practices were found to be mostly composed of *Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Acidobacteria*, which differed in abundance depending upon the growth stage of the fields. Only a few previous studies have compared bacterial communities in conventional and no-tillage fields of major crops (Drijber *et al.*, 2000; Feng *et al.*, 2003). Our results support some previous studies that the bacterial communities were different at certain growth stages of rice. In particular, there was always an increasing or decreasing trend from the pre-sowing to ripening stage (Reichardt *et al.*, 1997). However, previous studies did not determine which types of bacteria were present during the different growth stages of rice except by groups such as spore-forming and denitrifying bacteria (Reichardt *et al.*, 1997). In this study, various groups of bacteria were cultured and identified. A group of *Actinobacteria* remained almost constant from the zero to vegetative phase but decreased thereafter during the reproductive and ripening stages in both field soils. Ghoshal and Singh (1995) indicated that the levels of microbial biomass decreased sharply from the seedling to the flowering stage and then increased slightly with crop maturity, which supports the variation of certain bacterial groups in our results. This might be due to changes in nutrient balance such as total nitrogen, carbon, or phosphorus, or pH of the soil, due to the different soil cultivation methods (Table 1) (Barrett and Burke, 2000).

Among the 132 bacterial strains isolated from both field soils, 6% of the strains had 16S rRNA gene sequence similarity <96%, indicating presumably novel bacteria that were most closely related with previously uncultured bacteria (Ward *et al.*, 1990). One bacterial strain T2-YC6790^T, which was strongly associated with *Actinobacteria* was present in both samples at different growth stages. Four other interesting strains, T2-YC6788, Z0-YC6835, Z0-YC6837, and Z2-YC6860, with low 16S rRNA gene sequence similarity (88.5–93.7%) to validly published strains, require further study regarding their novelty based on taxonomic characterization. Principal coordinates analysis of unweighted UniFrac distances revealed significant differences between all growth stages in both fields and clearly grouped all samples separately, suggesting that the two rice fields had specific bacterial populations within each cultivation practice at different growth stages (Lozupone *et al.*, 2006).

No culturable *Actinobacterial* strains were observed in Z2 and T3 soils, but there appeared to be abundant levels of *Actinobacteria* distributed in all stages of rice cultivation in the metagenomic libraries. This shows that there are still many groups of bacteria in the rhizosphere that cannot be cultured (Ward *et al.*, 1990; Zengler *et al.*, 2002). The amplified metagenomes prepared from both field soils using actinobacterial-specific primers revealed a new group of bacteria in the *Gemmatimodadetes* that did not belong to *Actinobacteria* (Stach *et al.*, 2003). Other clones belonged to different species of *Streptomyces* and to *Actinocatenispora thailandica* TT2-10^T (Thawai *et al.*, 2006). Therefore, constructing metagenomic libraries using actinobacterial- and streptomycete-specific primers can identify more of the total richness of *Actinobacteria* in an experimental system. *Arthrobacter*, which was detected at the pre-sowing phase in both soils (Z0 and T0) and at the vegetative phase in T soil (T1) by the culture method, was detected at almost all stages in both soil managements in the metagenomic libraries. It was clear that better bacterial diversity data can be obtained by combining culture-dependent and independent methods (Kisand and Wikner, 2003).

We did not find any clones other than *Streptomyces* species due to the specific binding of streptomycete-specific primers (Rintala *et al.*, 2001; Babalola *et al.*, 2009). The screening of

metagenomic libraries using streptomycete-specific primers showed much higher diversity of *Streptomyces* species than the culture method in both field samples. Only two strains related to the genus *Streptomyces* were retrieved from the pre-sowing phase of both soils by culturing, but the clones from the metagenomic screening revealed that many *Streptomyces* species abundantly existed in the rice rhizosphere. The majority of the clones identified in the metagenomic study were related to uncultured actinobacterial species, which need improved cultivation methods for conducting experiments seeking to understand their possible role(s) in the rice rhizosphere.

The change in bacterial communities during different rice growth stages indicated that the changes were similar irrespective of field conditions, although diversity varied. It has been shown that plant species have a greater effect on the rhizosphere microflora than the plant's developmental stage (Wieland *et al*., 2001). We showed that developmental stage might also have a significant effect on the rice bacterial community. However, more studies are needed to verify microbial diversity and its role at different growth stages of rice and other crops.

There is also a need to explore group-specific and soil-specific diversity of bacteria in rice for better management (Garbeva *et al*., 2004). We conclude that tillage practices and crop growth stages are important when characterizing bacterial communities and both have a strong influence on bacterial diversity. Additionally, we succeeded in increasing the number of culturable bacteria from the paddy soil samples with modified cultivation techniques and isolated several novel lineages within a number of bacterial divisions. Additional culture-dependent and molecular methods are needed for a complete investigation of microbial diversity.

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